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SOLUTIA
Applied Chemistry, Creative Solutions

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57109

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March 5, 2002

TSCA Document Processing Center
US Environmental Protection Agency
EPA East Room 6428 (MC: 7407M)
1200 Pennsylvania Avenue, NW
Washington, DC 20460

Re: Notification of new information of substantial risk under TSCA Section 8(e)

Dear Sir/Madam:

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As stipulated in Section 8(e) of the Toxic Substances Control Act, Solutia Inc. is submitting the attached study:

Study Title: Chromosomal Aberration Study of Dequest 2066 in Cultured Mammalian Cells.

Solutia Study Number: MI20000047

Product Name: Dequest® 2066 Deflocculant and Sequestrant

CAS Number: 22042-96-2

Chemical Identity: Phosphonic acid, [[(phosphonomethyl) imino] bis[2,1-ethanediylnitrilobis(methylene)]] tetrakis-, sodium salt

The study was performed on behalf of Solutia Japan Limited at a testing facility in Japan.

The study examined the capacity for the test article to induce chromosomal aberrations in vitro in an established cell line (CHL/IU) from lungs of a female Chinese hamster. The study design conformed to OECD testing guidelines (#473) through pulse treatment and

for a 24-hour continuous exposure interval; a 48-hour exposure interval, not recommended by OECD but conforming to Japanese testing Guidelines on Industrial Chemicals, was also examined.

A dose-related increase in the incidence of structural, but not numerical, chromosomal aberrations was observed following the continuous 48-hour treatment. Following traditional study design, negative (no activity) results had been obtained following pulse treatment with and without metabolic activation, as well as after 24 hours of continuous treatment. Thus, a positive response was observed only during that portion of the study not included in internationally accepted testing guidance. Regarding this study extension, we note the opposition "to treatment lengths as long as 48 hours" cited in the Report of the OECD Working Group on in vitro tests for chromosomal aberrations (Mut. Res. 312:241-261; 1994) "because of the possibility of false positive results arising for a variety of reasons...".

Please contact me if you have any questions.

Sincerely,

James E. Downes
Product Stewardship
Industrial Products

Direct Telephone: 314-674-2918

Confirmatory Statement of the Study Report

General Testing Research Center,

Japan Oilstuff Inspectors' Corporation

Sponsor : Solutia Japan Limited

Title : Chromosomal Aberration Study of DEQUEST 2066 in Cultured Mammalian Cells

Study No. : B001157

The study has been conducted in General Testing Research Center, Japan Oilstuff Inspectors' Corporation. The original study report was written in Japanese and translated into English language. I hereby declare that this report reflects faithfully the original study report as much as possible to my knowledge.

Translator : M. Nakamura Date: Feb. 12, 2002

Masato Nakamura

Submitted to :

Solutia Japan Limited

Report

Chromosomal Aberration Study of DEQUEST 2066 in Cultured Mammalian Cells

(Study No. : B001157)

MI20000047

October 18, 2001

GENERAL TESTING RESEARCH CENTER, JAPAN OILSTUFF INSPECTORS' CORPORATION

Statement

General Testing Research Center,

Japan Oilstuff Inspectors' Corporation

Sponsor : Solutia Japan Limited

Title : Chromosomal Aberration Study of DEQUEST 2066
in Cultured Mammalian Cells

Study No. : B001157

The study in this report was conducted in compliance with the following Good Laboratory Practice Standards.

「Japanese GLP standards Applied to Industrial Chemicals (Kanpogyo No. 39, Yakuhatsu No. 229, 59 Kikyoku No. 85, 1984; revised in 1988 and 2000)」

「OECD Principles of Laboratory Practice (1997) 」

Management : Masakatsu Usami sealed Date: October 18, 2001

Quality Assurance Statement

General Testing Research Center,

Japan Oilstuff Inspectors' Corporation

Sponsor : Solutia Japan Limited

Title : Chromosomal Aberration Study of DEQUEST 2066 in Cultured Mammalian Cells

Study No.: B0001157

By the inspections and audits listed below, i hereby certify that the study was carried out in accordance with its protocol and with our Standard Operating Procedures, and that the report faithfully describes the methods and procedures applied, and that the reported results reflect the raw data of the study accurately.

Inspection or audit	Date of inspection or audit	Date of reporting to the study director and to the management
Study Protocol	June 7, 2001	June 7, 2001
Study Procedure	July 6, 2001 August 21, 2001	July 6, 2001 August 21, 2001
Draft Report	October 1, 2001	October 1, 2001
Study Report	October 18, 2001	October 18, 2001

Outline of the Study

1. Title : Chromosomal Aberration Study of DEQUEST 2066 in Cultured Mammalian Cells (Study No.:B001157)

2. Purpose : To assess the clastogenicity of the test substance by the chromosomal aberration tests using cultured mammalian cells.

3. Guideline : Japanese Guidelines on Industrial Chemicals (1997), OECD Guideline (1997)

4. GLP : Japanese GLP on Industrial Chemicals (1984; revised in 1988 and 2000) and OECD GLP(1997)

5. Sponsor : Solutia Japan Limited
(Shinkawasanko Bldg. 2F 1-3-17, Shinkawa, Chuo-ku, Tokyo)
(Responsible person) Noboru Watanabe

6. Organization in Contractor : Mitsubishi Chemical Safety Institute Ltd.
(1-30, 2-Chome, Shiba, Minato-ku, Tokyo)

7. Testing Facility : General Testing Research Center,
Japan Oilstuff Inspectors' Corporation
(10-4, 1-Chome, Mikagetsukamachi, Higashinada-ku,
Kobe-shi, Hyogo)

8. Study Contributors

Management : Masakatsu Usami

Responsibility person of data keeping : Etsurou Fujiwara

Study Director : Masato Nakamura sealed Date: October 18, 2001

(17-7-319, 6-Chome, Symiyoshimiyamachi, Higashinada-ku, Kobe-shi, Hyogo)

Other Contributor : Osamu Iwaihara

9. Study Date : (Initiation of the Study) June 7, 2001

(Initiation of the Test) June 15, 2001

(Submission of the Test) September 18, 2001

(Submission of the Final Report) October 18, 2001

10. Data analysis : No statistical methods were used.

11. There was no unforeseeable circumstances that had affected test results undesirably.

12. Retention : All records will be retained in the safekeeping facility of General Testing Research Center for 10 years after the submission of the final report. Further retention will be discussed with the sponsor.

Contents

Summary

A chromosomal aberration study of DEQUEST 2066 *in vitro* was made with an established cell line (CHL/IU) from the lungs of a female Chinese hamster.

First, the pulse treatment test was executed, with and without metabolic activation, and the incidences of structural chromosomal aberration or numerical aberration were less than 5% in all treatment groups.

From the negative results of pulse treatment test, a continuous test with 24 hours treatment was executed. The incidences of structural chromosomal aberration or numerical aberration were less than 5% in all treatment groups.

The remarkable delay of the cell cycle by the test substance is observed in continuous test with 24 hours treatment. Therefore 48 hours treatment (3.0 cell cycle) was executed.

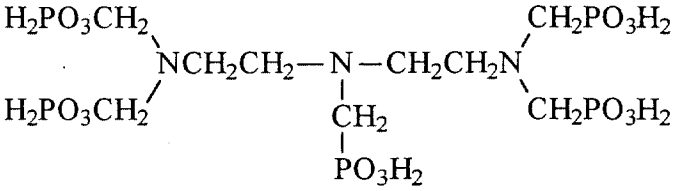
In the chromosomal aberration test with 48 hours treatment, the incidences of structural chromosomal aberration were 17.0% at 150 μg/ml, 37.0% at 200 μg/ml and 48.5% at 300 μg/ml in 48 hours treatment. The incidences of numerical aberration (polyploid cell) were less than 5% in all treatment groups.

From these findings, it was concluded that DEQUEST 2066 has a clastogenic potential in the CHL/IU cell line.

Materials and Methods

1. Test materials

1.1 Test substance

Name of the new chemical substance (IUPAC nomenclature)	Sodium salt of diethylenetriamine (tri- penta) (methylenephsphonic acid)		
Trade name	DEQUEST 2066		
Structural formula or rational formula (or outline of manufacturing method, in case both are unknown)	Main component Sodium salt of diethlylenetriamine pentamethylenephoshonic acid (23.7%)  Sodium salt of diethlylenetriamine trimethylenephoshonic acid (4.2%) Sodium salt of diethlylenetriamine tetramethylenephoshonic acid (1.0%)		
Purity of the new chemical substance tested	Total of above three components 28.9 %	Lot No. of the new chemical substance to be tested	OE460
Name and concentration of impurities	Sodium phosphite 1.9 %, Sodium chloride 5.0 %, Sodium orthophosphite 0.48 %, others 0.72 %, Water 63.0 %		
CAS No.	22042-96-2	Vapor pressure	———
Molecular weight	———	Partition coefficient	———
Melting point	———	Appearance at ambient temperatures	Darkbrown liquid
Boiling point	105℃		
Stability	Stable		

Solubilities in several solvents	Solvent	Solubilities	Stability in Solvent
	Water(saline)	soluble at 50 mg/ml [1)]	stable[2)]
	DMSO	———	———
	Acetone	———	———
	others(——)	———	———

DMSO : dimethylsulfoxide

Storage condition : kept at a room temperature, darkplace

1) : According to examination of vehicle.

2) : Heat of dissolution, discoloring and foam formation were not observed during preparation for dissolving the test substance to be tested.

1.2 Preparation of test

1) Negative control material (solvent)

Saline (Otsuka Pharmaceutical, Lot No. MOK92)

2) Solvent selection and preparation for making solution of test substance

The examination of vehicle was conducted based on the information in which the test substance was soluble in water. From the examination of vehicle, saline was used as the solvent for the test substance. The test substance solution was prepared just before use.

At the preparation of the test solution, purity compensation was performed based on the purity 28.9 %.

The test substance solution presents from sponsor was dissolved in saline, and diluted into each concentration.

1.3 Positive material

1) Positive controls

Mitomycin C (MMC, Lot No. KSM4520, purity 99.8%, Wako Pure Chemical Laboratory Co., Ltd.)

Benzo[a]pyrene (BP, Lot No.GG01, purity 95.6%, Tokyo Kasei Kogyo Co., Ltd.)

2) Positive control solutions

MMC was dissolved in saline (Otsuka Pharmaceutical Factory, Inc.) to a concentration of 1.5 μ g/ml before use. BP was dissolved in DMSO (Dojin Chemical Laboratory Co., Ltd.) to 4 mg/ml, frozen, and thawed at the room temperature before use.

2. Indicator cell line

The CHL/IU cell line, originally derived from the lung of a female Chinese hamster, was used. The cells were purchased from Dainippon Pharmaceutical Co., Ltd. A cell suspension was mixed with one tenth volume of DMSO, and then frozen in 1 ml and stored in liquid nitrogen. The suspended cells were used after thawing and cultivation through up to 5 passages. The cells were cultured in plastic

CO_2 incubator (Tabai Espec Corp. model BNA-111A, 121DA; CO_2 5%, temperature 37 ℃, humid condition).

The final doubling time of the cell used for the test was about 16.4 hours.

3. Culture media

3.1 Eagle's minimum essential medium

Eagle's minimum essential medium (MEM; Eagle's MEM "Nissui ①", Nissui Pharmaceutical Co., Ltd.) of 8.3g was dissolved in 880 ml of distilled water and autoclaved at 121 ℃ for 15 min. The solution was supplemented with 8.8ml of sterilized 2.92% L-glutamine solution and 11.2 ml of sterilized 10% sodium bicarbonate solution.

3.2 Growth medium

Nine hundred ml of the above MEM solution was supplemented with 100 ml of heat-inactivated (56 ℃, 30 min.) calf serum (GIBCO BRL; Lot No. 296130).

4. S9 mix

4.1 S9

S9 from Kikkoman Corporation was a supernatant fraction of liver homogenate prepared from rats which were given i.p. injection of phenobarbital at 30, 60, 60 and 60 mg/kg every day and 5,6-benzoflavone at 80 mg/kg at the third PB injection. The S9 was stored below -80 ℃.

Lot No.	RAA-444
Prepared on	April 27, 2001
Using animal Species, Strain	Sprague-Dawley Rat
Sex, Age (in weeks)	Male, 7 weeks old
Weight	205 – 241

Final concentration of the S9 was 5%, protein of S9 was 1.2825 mg/ml.

4.2 S9 mix

The composition of S9 mix is listed in the following table. S9 mix was freshly prepared for each assay, and was stored in the ice.

Components	Amount in 1 ml
S9	0.3 ml
$MgCl_2 \cdot 6H_2O$	5 μmol
KCl	33 μmol
D-glucose-6-phosphate	5 μmol
β-$NADP^+$	4 μmol
HEPES buffer (pH 7.2)	4 μmol
Sterilized distilled water	Remainder

5. Test Method

5.1 Pulse treatment test

1) Cell growth inhibition test

(1)Test substance concentration

Six dose levels ranging from 156 to 5000 μg/plate was graded at a common ratio of 2.

(2)Treatment of cells

In a 6 cm plastic plate, 5 ml of cell suspension (4×10^3/ml) were incubated for 3 days. After removal of the culture medium from each plate, the following table shows the composition, treatment of cells was performed for 6 hours and the tests were made using two plates for each concentration. After 6 hr. incubation, the cells were washed three times with MEM, and incubated in 5 ml of a fresh growth medium for a further 18 hr.

In the negative control group, cells were treated with vehicle.

	test substance solutions and negative control	S9 mix	the growth medium
without metabolic activation	0.3 ml	—	2.7 ml
with metabolic activation	0.3 ml	0.5 ml	2.2 ml

(3)Measurement of surviving cells

Cells were washed with Dulbecco's phosphate buffered saline (PBS(-); Dulbecco's PBS "Nissui", Nissui Pharmaceutical Co., Ltd.), fixed with methanol for 15 min., stained with 0.1% crystal violet solution for 10 min., and washed with water and dried. Cell density was measured with a cell density counter(Monocellator, Olympus Co., Ltd.).

(4)Measurement of 50% inhibition concentration of cell growth

The ratio of surviving cells in treatment groups were calculated in comparison with negative control. Then, a survival curve was drewed, and 50% inhibition concentration of cell growth (IC_{50}) was calculated.

2) Chromosomal aberration test

(1)Test substance concentration

Fifty percent inhibition concentration of cell growth was not obtained without and with metabolic activation.

Without metabolic activation

concentration (μg/ml)	negative control	156	313	625	1250	2500	5000
cell growth (%)	100	109	109	97	96	76	58

With metabolic activation

concentration (μg/ml)	negative control	156	313	625	1250	2500	5000
cell growth (%)	100	98	104	103	108	102	77

The concentrations were set at 5000, 2500, 1250 and 625 μg/ml without and with metabolic activation based on the result of cell growth inhibition tests.

In positive controls, final concentrations of MMC and BP were set at 0.10 and 15 μg/ml, respectively, which are the concentration levels as known to induce chromosomal aberration.

(2)Treatment of cells

The cultured cells were treated with the test substance using the same procedure as described above.

For positive controls, the cells were treated with a mixture of 2.7 ml of the growth medium and 0.3 ml MMC solution in the treatment without metabolic activation and treated with a mixture of 2.5 ml of the growth medium and 0.5 ml of S9 mix and 15 μl BP solution in the treatment with metabolic activation.

(3)Preparation of specimens and measurement of surviving cells

Cell density was measured in the preparation of specimens. The cultured cells were treated with the test substance using the same procedure as described above. Before 2 hours from the end of the treatment, colcemid was added to the medium in each plate to a final concentration of 0.1μg/ml to arrest cells in metaphase during preparing specimen. The cells were then washed with PBS(-), dissociated with 0.25% trypsin solution, and centrifuged (1000 rpm, 5 min.; same below) for collecting them. After removal of the supernatant, 4 ml of 0.075 M potassium chloride solution was added for hypotonic treatment (37 ℃, 15 min.). The cells were then fixed in 0.5 ml of cold methanol/acetic acid mixture (3/1, v/v). After their centrifugation, the supernatant was removed and 4 ml of a fresh fixative added. The fixation procedure was repeated 3 to 4 times. Thereafter, the cells were suspended in a small amount of the fixative, and drops of the cell suspension were placed in two positions on a slide glass and dried. The cells were stained for 20 min. with 3% Giemsa's solution diluted with 1/15 M phosphate buffer (pH 6.8). The slides were then washed with water, dried, and coated with a mounting medium. Three slides were prepared for each plate.

(4)Observation

①Preliminary observation

Each specimen was observed first to confirm that it has an adequate (50 cells for one plate) number of mitotic cells. For positive and negative controls, the incidence of structural aberrations was confirmed to be appropriate.

②Structural aberration and numerical aberration

Each specimen was coded, 100 metaphase cells for each plate (i.e. 200 cells

Metaphase cells of which chromosomes are spread well were selected and observed for structural aberrations. Cells without aberration but not having 25±2 chromosomes were omitted from the survey.

Structural aberrations were classified as follows[1]:

chromatid breaks (ctb)

chromatid exchanges (cte)

chromosome breaks (csb)

chromosome exchanges (cse, dicentric, ring, etc.)

other aberrations (frg)

A "gap" was distinguished from a "break" as follows. The "gap" is an achromatic region in a single chromatid which is narrower than the width of the chromatid. Gaps are not included to the chromosomal aberration. The recorded gaps were distinguished from that of the other structural aberration.

Numerical aberration was observed and polyploid cells, which include endoreduplicated cells, were scored.

③Mitotic index

One thousand cells from each plate (i.e. 2000 cells for each concentration) were observed to calculate mitotic index.

(5) Judgment

A cell with at least one structural chromosomal aberration was classified as aberrant cell. The number of aberrant cells was counted in different kinds of two ways, one includes cells with no aberration other than gaps (+gap), and another excludes such above cells (-gap).

The chromosomal aberration potential of the test substance was judged as follows. When both of incidence of -gap aberrant cells and that of numerical aberrations are less than 5%, the substance is negative (-). When either of incidences is 5% or more and less than 10%, the test substance is inconclusive (±). When either of incidences is 10% or more, the test substance is positive (+). When the chromosomal aberration test is inconclusive or positive, the

concentration of confirmation examination is decided according to the incidence of the chromosome aberration. According to case, examine by the equal ratio. It is judged positive in the case where reproducibility is admitted.

5.2 Taking data

Each of the sums and the incidence (%) of the chromosome aberration and numerical aberrations was shown. The chromosomal aberration was shown dividing the number of cells in the type. Each of Cell growth index in cell growth inhibition tests and chromosome aberration tests were shown. When the test was positive, the "D_{20}" values were calculated from the test results. The "D_{20}" value is the concentration (mg/ml) required to induce any aberration in 20% of metaphases. The microscopic photograph of a typical chromosomal aberration is appended.

5.3 Continuous test

A continuous test with 24 hours treatment was executed. Because the result of the pulse treatment method was negative(1.5 cell cycle). The delay of the cell cycle by the test substance is observed. So we judged that the more than longer treatment than 24 hours treatment was necessary, and 48 hours treatment (3.0 cell cycle) was executed.

(1)Test substance concentration

Six dose levels ranging from 156 to 5000 μg/plate was graded at a common ratio of 2.

Fifty percent inhibition concentration of cell growth was calculated to be a 442 μg/ml at 24 hours treatment and 158 μg/ml at 48 hours treatment in the cell growth inhibition tests.

24 hours treatment

concentration (μg/ml)	negative control	156	313	625	1250	2500	5000
cell growth (%)	100	115	81	47	37	19	0

48 hours treatment

concentration (μg/ml)	negative control	156	313	625	1250	2500	5000
cell growth (%)	100	50	25	7	5	1	0

The concentrations were set at 3000, 2400, 1800, 1200, 600, 300 and 150 μg/ml with 24 hours treatment, and were set at 400, 300, 200, 150, 100 and 50 μg/ml with 48 hours treatment based on the result of cell growth inhibition tests.

In the chromosomal aberration test of 24 hours treatment, the delay of the cell cycle by the test substance is observed, metaphase had not obtained more than 50 cells at most low dose 150 μg/ml.

The result of cell growth inhibition test 24 hours treatment.

24 hours treatment of the chromosomal aberration test

concentration (μg/ml)	negative control	150	300	600	1200	1800	2400	3000
cell growth (%)	100	93	79	53	49	34	25	20

The concentrations were set at 300, 150, 75, 37.5, 18.8, 9.4 and 4.7 μg/ml with 24 hours treatment based on the result of chromosomal aberration test Ⅰ.

In positive control, final concentrations of MMC was set at 0.03μg/ml, the concentration levels in which was known to induce chromosomal aberration.

(2)Treatment of cells

In a 6 cm plastic plate, 5 ml of cell suspension (4×10^3/ml) were incubated for 3 days. After removal of the culture medium from each plate, the following table shows the composition, treatment of cells was performed for 24 hours and the test was performed using two plates for each concentration. For positive controls, the cell were treated with a mixture of 5.0 ml of the growth medium and 0.05 ml of MMC solution.

	test substance solutions and negative control	the growth medium
24 hours treatment (1.5 cell cycle)	0.5 ml	4.5 ml
48 hours treatment (3.0 cell cycle)	0.5 ml	4.5 ml

Preparation of specimens, observation and judgment using same procedure as described above.

Results and Discussion

In the cell growth inhibition tests of pulse treatment test, fifty percent inhibition concentration of cell growth was not obtained without and with metabolic activation(Figure 1, 2). On the basis of the cell growth inhibition tests, the concentrations were set at 5000, 2500, 1250 and 625 μg/ml without and with metabolic activation.

In the cell growth inhibition test and the chromosomal aberration test of pulse treatment, precipitated test substance was not observed in the medium before and after incubation.

In the chromosomal aberration test of pulse treatment test, the incidences of structural chromosomal aberration and numerical aberration were less than 5% in all treatment groups both of without and with metabolic activation. The incidences of structural aberrations in the positive control groups were significantly higher than ones in negative control groups(Table 1 and Figure 3, 4).

From the negative results of pulse treatment test, a continuous test with 24 hours treatment(1.5 cell cycle) and 48 hours treatment(1.5 cell cycle) was executed.

In the continuous test, 1.5 cell cycle was assumed to be 24 hours from the doubling time (16.4 hours) of used cell.

Fifty percent inhibition concentration of cell growth was calculated to be a 442 μg/ml at 24 hours treatment and 158 μg/ml at 48 hours treatment in the cell growth inhibition tests(Figure 5, 7).

The concentrations were set at 3000, 2400, 1800, 1200, 600, 300 and 150 μg/ml at on 24 hours treatment, and were set at 400, 300, 200, 150, 100 and 50 μg/ml at on 48 hours treatment based on the result of cell growth inhibition tests.

In the cell growth inhibition test and the chromosomal aberration test Ⅰ and Ⅱ of the continuous test, precipitated test substance was not observed in the medium before incubation and after incubation.

In the chromosomal aberration test of 24 hours treatment, the delay of the cell

cells at most low dose 150 μg/ml. Therefore the chromosomal aberration test was executed again, the concentrations were set at 300, 150, 75, 37.5, 18.8, 9.4 and 4.7 μg/ml at on 24 hours treatment, the test was made the chromosomal aberration test Ⅱ.

In the chromosomal aberration test Ⅱ of continuous treatment at 24 hours treatment, the inhibition of the cell growth by the test substance is observed and metaphase had not obtained more than 50 cells at 300 μg/ml for delay of the cell cycle.

In the chromosomal aberration test Ⅰ of continuous treatment at 48 hours treatment, the delay of the cell cycle by the test substance is observed, metaphase had not obtained more than 50 cells at 400 μg/ml for cell growth inhibition.

In the chromosomal aberration test Ⅰ and Ⅱ of continuous treatment at 24 hours treatment, the incidences of structural chromosomal aberration and numerical aberration were less than 5% in all treatment groups. In the chromosomal aberration test Ⅰ of continuous treatment at 48 hours treatment, the incidences of numerical aberration were less than 5% in all treatment groups and, the incidences of structural chromosomal aberration were 17.0% at 150 μg/ml, 37.0% at 200 μg/ml and 48.5% at 300 μg/ml. The incidences of structural aberrations in the positive control groups were significantly higher than those in negative control groups (Table 2, Figures 8, 9, 10 and 11).

D_{20} value was calculated from the treatment group with the increase in structural chromosomal aberration cell, and was 0.14 mg/ml at 48 hours treatment.

Microscopic photographs of normal cell for the negative control and structural cell in 48 hours treatment are shown in photo. 1 and 2.

Structural chromosomal aberration was judged positive in continuous treatment at 48 hours treatment and the examination was assumed to be an end.

No statistical methods was used for data analysis.

From these findings, it was concluded that DEQUEST 2066 has a clastogenic potential in a CHL/IU cell line.

Reference

1) Mammalian Mutagenicity Study Group of the Environmental Mutagen Society of Japan : Atlas of chromosomal aberrations induced by chemicals, Asakura Press, Tokyo, 1988.

Table 1 Result of the chromosomal aberration test (pulse treatment test)

Test substance : DEQUEST 2066

Treatment time (h)	S9mix	Test substance dose levels (μg/ml)	Number of cells with chromosomal structural aberrations								Number of cells with gaps	Cell growth index (%)	Number of cells with numerical aberrations			
			Number of cells analyzed	Chromatid breaks	Chromatid exchanges	Chromosome breaks	Chromosome exchanges	Other aberration *	Number of cells total aberrations (%)				Number of cells analyzed	Polyploids	Others **	Number of cells total aberrations (%)
6-18	-	Negative control (Saline)	100	0	0	0	0	0	0		1	100	100	0	0	0
			100	0	0	0	0	0	0		0		100	0	0	0
			200	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)		1 (0.5)		200	0 (0.0)	0 (0.0)	0 (0.0)
6-18	-	625	100	1	0	0	0	0	1		0	98	100	0	0	0
			100	0	0	0	0	0	0		0		100	0	0	0
			200	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)		0 (0.0)		200	0 (0.0)	0 (0.0)	0 (0.0)
6-18	-	1250	100	2	0	0	0	0	2		0	91	100	0	0	0
			100	1	1	0	0	0	2		0		100	0	0	0
			200	3 (1.5)	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	4 (2.0)		0 (0.0)		200	0 (0.0)	0 (0.0)	0 (0.0)
6-18	-	2500	100	2	1	0	0	0	3		2	71	100	0	0	0
			100	4	0	0	0	0	4		2		100	2	0	2
			200	6 (3.0)	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	7 (3.5)		4 (2.0)		200	2 (1.0)	0 (0.0)	2 (1.0)
6-18	-	5000	100	5	1	0	0	0	5		3	41	100	0	0	0
			100	4	0	0	0	0	4		0		100	0	0	0
			200	9 (4.5)	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	9 (4.5)		3 (1.5)		200	0 (0.0)	0 (0.0)	0 (0.0)
6-18	-	Positive control (MMC) 0.10	100	30	59	0	0	0	69		0	/	100	0	0	0
			100	24	62	0	0	0	70		1		100	0	0	0
			200	54 (27.0)	121 (60.5)	0 (0.0)	0 (0.0)	0 (0.0)	139 (69.5)		1 (0.5)		200	0 (0.0)	0 (0.0)	0 (0.0)
6-18	+	Negative control (Saline)	100	0	0	1	0	0	1		0	100	100	0	0	0
			100	0	0	0	0	0	0		0		100	0	0	0
			200	0 (0.0)	0 (0.0)	1 (0.5)	0 (0.0)	0 (0.0)	1 (0.5)		0 (0.0)		200	0 (0.0)	0 (0.0)	0 (0.0)
6-18	+	625	100	0	0	0	0	0	0		0	91	100	0	0	0
			100	0	2	0	0	0	2		1		100	0	0	0
			200	0 (0.0)	2 (1.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (1.0)		1 (0.5)		200	0 (0.0)	0 (0.0)	0 (0.0)
6-18	+	1250	100	0	0	0	0	0	0		0	95	100	0	0	0
			100	0	1	0	0	0	1		0		100	0	0	0
			200	0 (0.0)	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)		0 (0.0)		200	0 (0.0)	0 (0.0)	0 (0.0)
6-18	+	2500	100	1	1	0	0	0	2		0	99	100	0	0	0
			100	2	2	0	0	0	2		0		100	0	0	0
			200	3 (1.5)	3 (1.5)	0 (0.0)	0 (0.0)	0 (0.0)	4 (2.0)		0 (0.0)		200	0 (0.0)	0 (0.0)	0 (0.0)
6-18	+	5000	100	1	0	0	0	0	1		0	67	100	0	0	0
			100	0	0	0	0	0	0		0		100	0	0	0
			200	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)		0 (0.0)		200	0 (0.0)	0 (0.0)	0 (0.0)
6-18	+	Positive control (BP) 15	100	4	22	0	0	0	24		0	/	100	0	0	0
			100	3	20	0	1	0	22		1		100	0	0	0
			200	7 (3.5)	42 (21.0)	0 (0.0)	1 (0.5)	0 (0.0)	46 (23.0)		1 (0.5)		200	0 (0.0)	0 (0.0)	0 (0.0)

Test substance treatment time (6 hours) - recover time (18 hours)

* : Others in structural aberration : fragmentation (except pulverization), ** : Others in numerical aberration : endoreduplicated cells

MMC : Mitomycin C, BP : Benzo (a) pyrene

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Table 2 Result of the chromosomal aberration test (continuous test)

Test substance : DEQUEST 2066

Treatment time (h)	Test substance dose levels (μg/ml)	Number of cells with chromosomal structural aberrations: Number of cells analyzed	Chromatid breaks	Chromatid exchanges	Chromosome breaks	Chromosome exchanges	Other aberrations *	Number of cells total aberrations (%)	Number of cells with gaps	Cell growth index (%)	Number of cells with numerical aberrations: Number of cells analyzed	Polyploids	Others **	Number of cells total aberrations (%)
24-0	Negative control (Saline)	100	1	0	0	0	0	1	0	100	100	0	0	0
		100	1	0	0	0	0	1	1		100	0	0	0
		200	2 (1.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (1.0)	1 (0.5)		200	0 (0.0)	0 (0.0)	0 (0.0)
24-0	4.7	100	0	0	0	0	0	0	1	101	100	0	0	0
		100	1	1	0	0	0	2	0		100	0	0	0
		200	1 (0.5)	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	2 (1.0)	1 (0.5)		200	0 (0.0)	0 (0.0)	0 (0.0)
24-0	9.4	100	2	0	0	0	0	2	0	102	100	0	0	0
		100	0	0	0	0	0	0	0		100	0	0	0
		200	2 (1.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (1.0)	0 (0.0)		200	0 (0.0)	0 (0.0)	0 (0.0)
24-0	18.8	100	1	0	0	0	0	1	1	104	100	0	0	0
		100	3	0	0	0	0	3	0		100	0	0	0
		200	4 (2.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (2.0)	1 (0.5)		200	0 (0.0)	0 (0.0)	0 (0.0)
24-0	37.5	100	2	2	0	0	0	4	0	107	100	0	0	0
		100	1	0	0	0	0	1	0		100	0	0	0
		200	3 (1.5)	2 (1.0)	0 (0.0)	0 (0.0)	0 (0.0)	5 (2.5)	0 (0.0)		200	0 (0.0)	0 (0.0)	0 (0.0)
24-0	75	100	2	0	0	0	0	2	1	103	100	0	0	0
		100	0	1	0	0	0	1	2		100	0	0	0
		200	2 (1.0)	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	3 (1.5)	3 (1.5)		200	0 (0.0)	0 (0.0)	0 (0.0)
24-0	150	100	4	0	0	0	0	4	2	113	100	0	0	0
		100	4	0	0	0	0	4	0		100	0	0	0
		200	8 (4.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	8 (4.0)	2 (1.0)		200	0 (0.0)	0 (0.0)	0 (0.0)
24-0	300	「 T O X 」***								93	「 T O X 」***			
24-0	Positive control (MMC) 0.03	100	14	17	0	0	0	30	0	/	100	0	0	0
		100	16	13	0	0	0	28	0		100	0	0	0
		200	30 (15.0)	30 (15.0)	0 (0.0)	0 (0.0)	0 (0.0)	58 (29.0)	0 (0.0)		200	0 (0.0)	0 (0.0)	0 (0.0)
48-0	Negative control (Saline)	100	1	0	0	0	0	1	0	100	100	0	0	0
		100	1	0	0	0	0	1	0		100	0	0	0
		200	2 (1.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (1.0)	0 (0.0)		200	0 (0.0)	0 (0.0)	0 (0.0)
48-0	50	100	2	0	0	0	0	2	0	99	100	0	0	0
		100	2	1	0	0	0	3	0		100	0	0	0
		200	4 (2.0)	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	5 (2.5)	0 (0.0)		200	0 (0.0)	0 (0.0)	0 (0.0)
48-0	100	100	5	2	0	0	0	7	0	68	100	0	0	0
		100	2	2	0	0	0	4	2		100	0	0	0
		200	7 (3.5)	4 (2.0)	0 (0.0)	0 (0.0)	0 (0.0)	11 (5.5)	2 (1.0)		200	0 (0.0)	0 (0.0)	0 (0.0)
48-0	150	100	17	7	0	0	0	19	0	57	100	0	0	0
		100	14	2	0	0	0	15	0		100	0	0	0
		200	31 (15.5)	9 (4.5)	0 (0.0)	0 (0.0)	0 (0.0)	34 (17.0)	0 (0.0)		200	0 (0.0)	0 (0.0)	0 (0.0)
48-0	200	100	36	3	0	0	1	38	1	43	100	0	0	0
		100	31	6	0	0	2	36	0		100	0	0	0
		200	67 (33.5)	9 (4.5)	0 (0.0)	0 (0.0)	3 (1.5)	74 (37.0)	1 (0.5)		200	0 (0.0)	0 (0.0)	0 (0.0)
48-0	300	100	47	4	0	0	6	53	0	20	100	0	0	0
		100	40	6	0	0	3	44	2		100	0	0	0
		200	87 (43.5)	10 (5.0)	0 (0.0)	0 (0.0)	9 (4.5)	97 (48.5)	2 (1.0)		200	0 (0.0)	0 (0.0)	0 (0.0)
48-0	400	「 T O X 」****								11	「 T O X 」****			
48-0	Positive control (MMC) 0.03	100	24	27	0	0	0	43	0	/	100	0	0	0
		100	23	29	0	0	0	43	0		100	0	0	0
		200	47 (23.5)	56 (28.0)	0 (0.0)	0 (0.0)	0 (0.0)	86 (43.0)	0 (0.0)		200	0 (0.0)	0 (0.0)	0 (0.0)

Test substance treatment time (24 hours) - recover time (0 hours)

* : Others in structural aberration : fragmentation (except pulverization), ** : Others in numerical aberration : endoreduplicated cells

MMC : Mitomycin C

「TOX」***: The delay of the cell cycle by the test substance is observed, metaphase cell had not obtained more than 50 cells

「TOX」****: The inhibition of the cell growth by the test substance is observed, metaphase cell had not obtained more than 50 cells

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Pulse treatment test

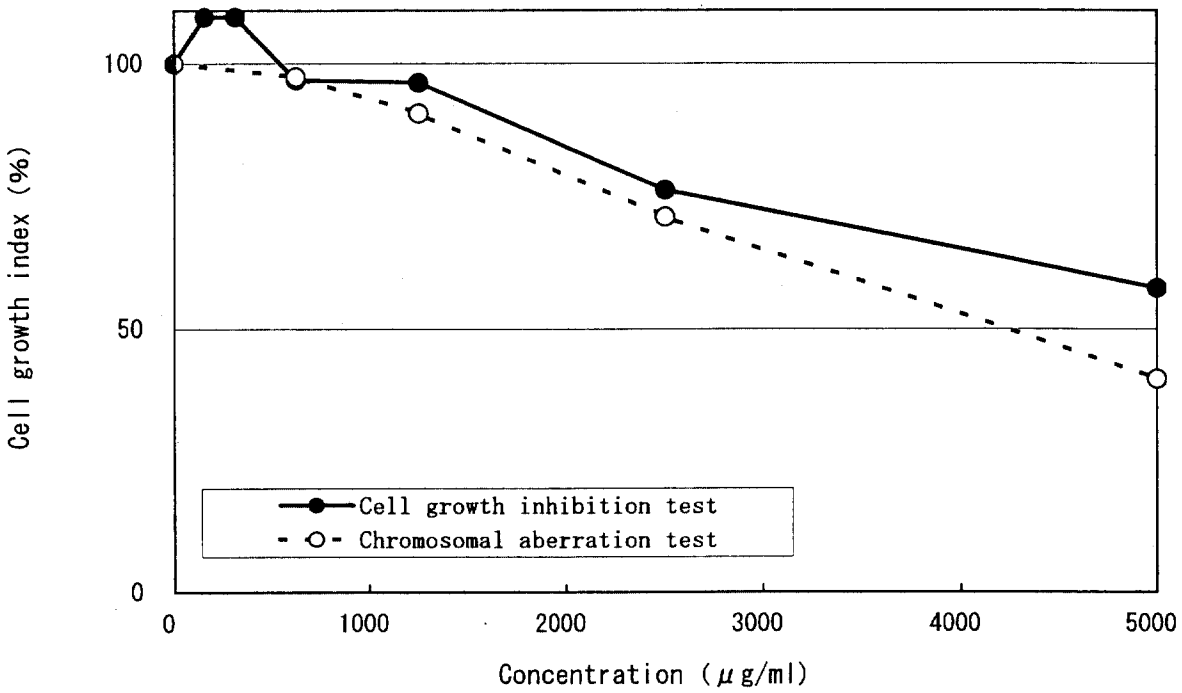

Fig. 1 Cell growth inhbition treated with DEQUEST 2066
(Without metabolic activation)

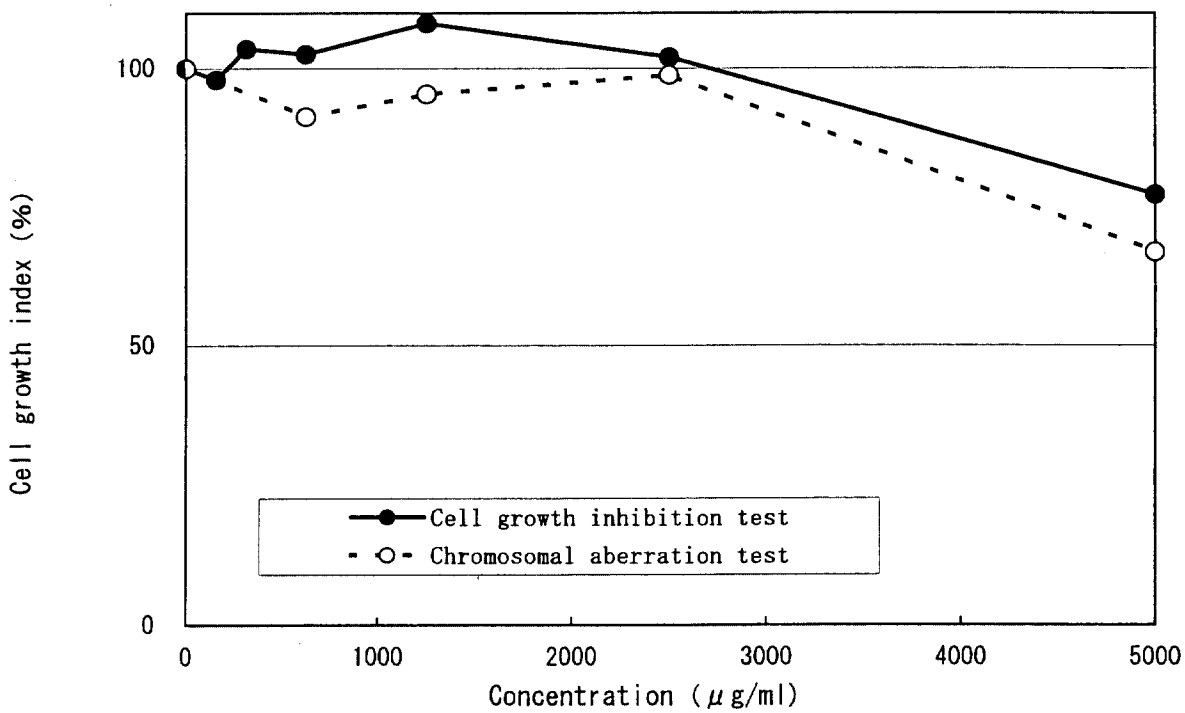

Fig. 2 Cell growth inhibition treated with DEQUEST 2066
(With metabolic activation)

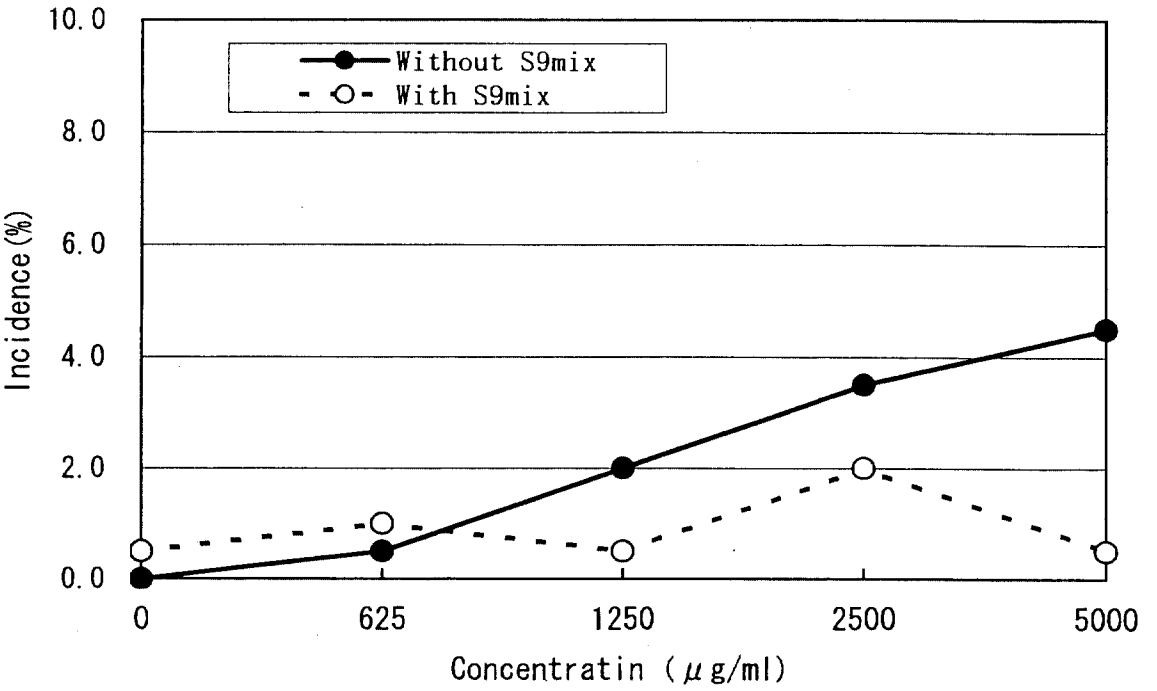

Fig. 3 Incidence of structural aberrent cell treated with DEQUEST 2066

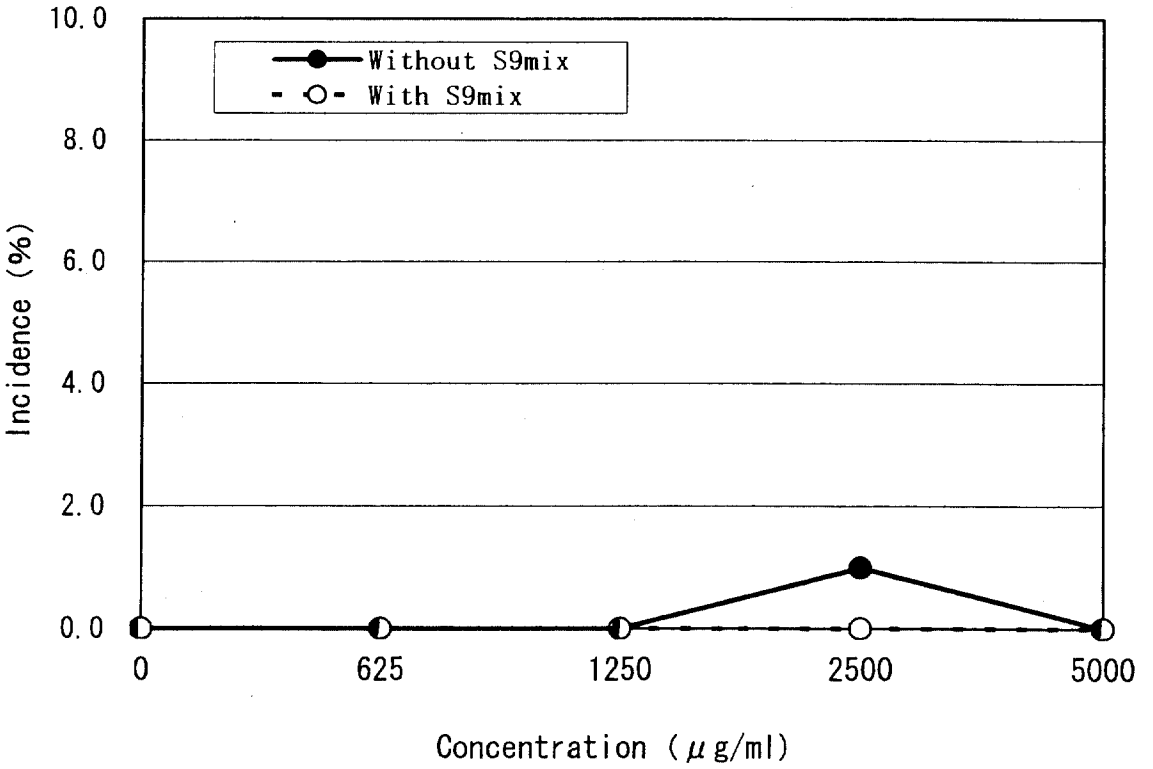

Fig. 4 Incidence of numerical aberrant cell treated with DEQUEST 2066

Continuous test

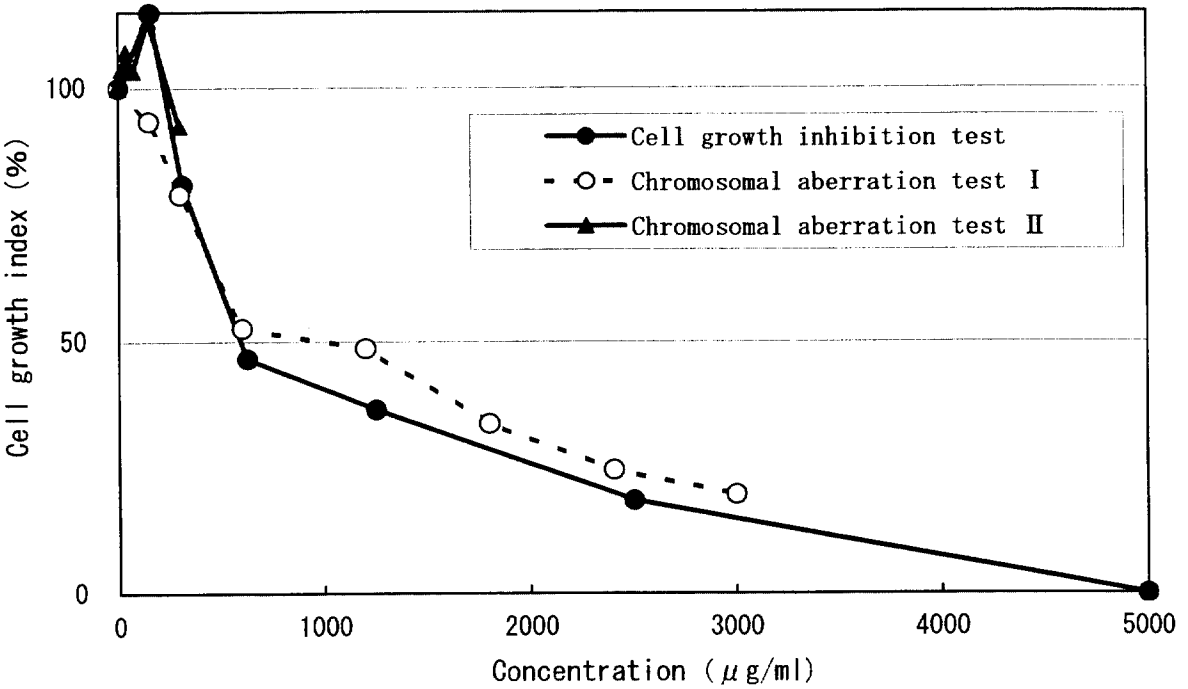

Fig. 5 Cell growth inhibition treated with DEQUEST 2066
(24 hours treatment)

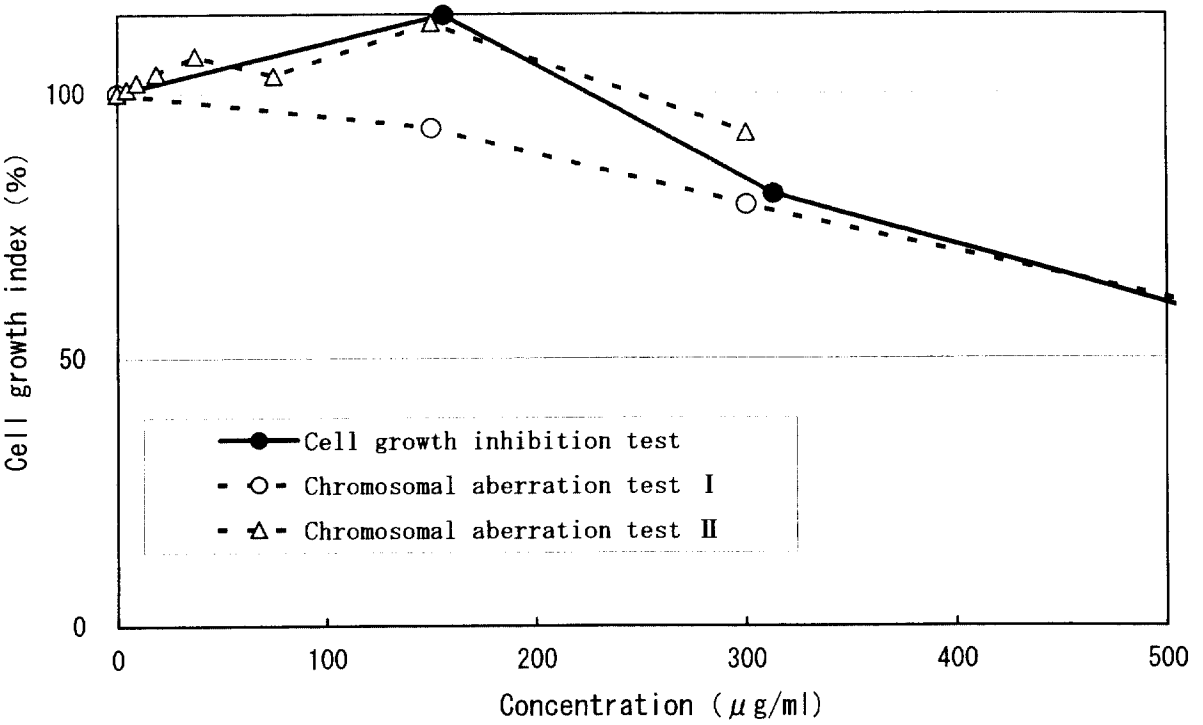

Fig. 6 Cell growth inhibition treated with DEQUEST 2066
(24 hours treatment)

In the chromosomal aberation test of 24 hours treatment, the delay of the cell cycle by the test substance is observed, metaphase had not obtained more than 50 cells at most low dose 150 μg/ml Therfore the chromosomal aberration test was executed again(Chromosomal aberration test II)

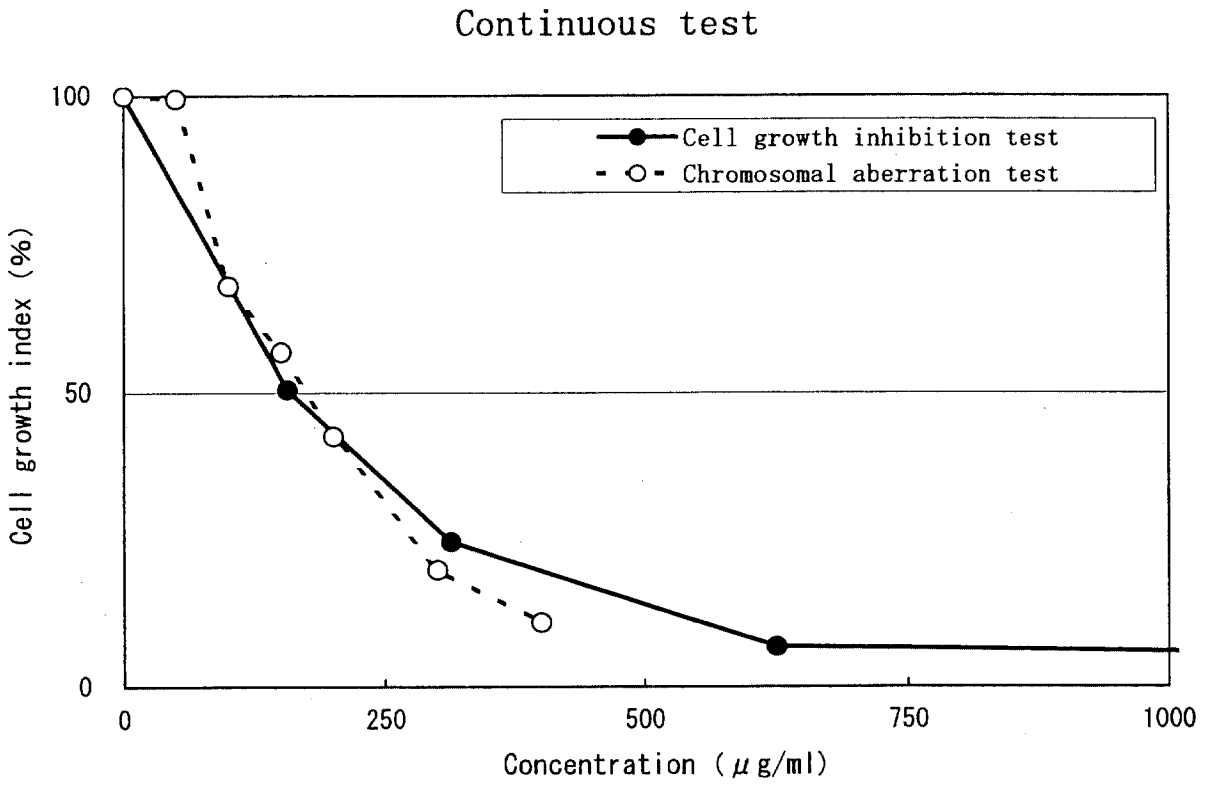

Fig. 7 Cell growth inhibition treated with DEQUEST 2066 (48 hours treatment)

Continuous test

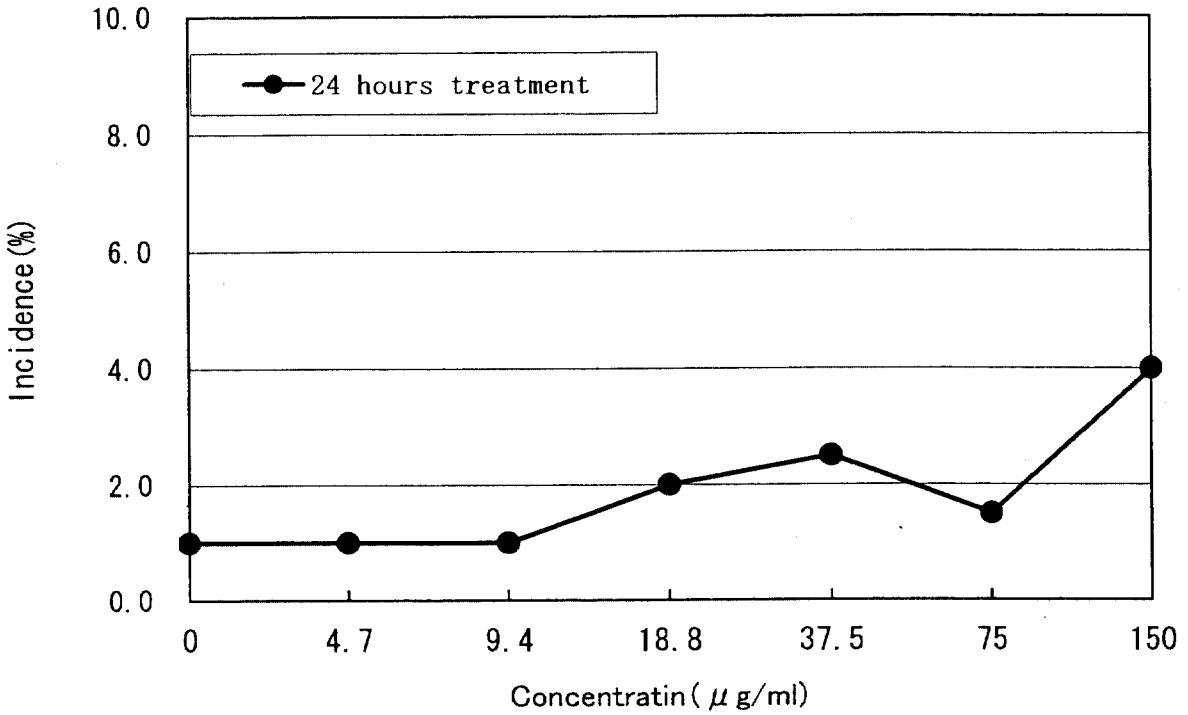

Fig. 8 Incidence of structural aberrent cell treated with DEQUEST 2066

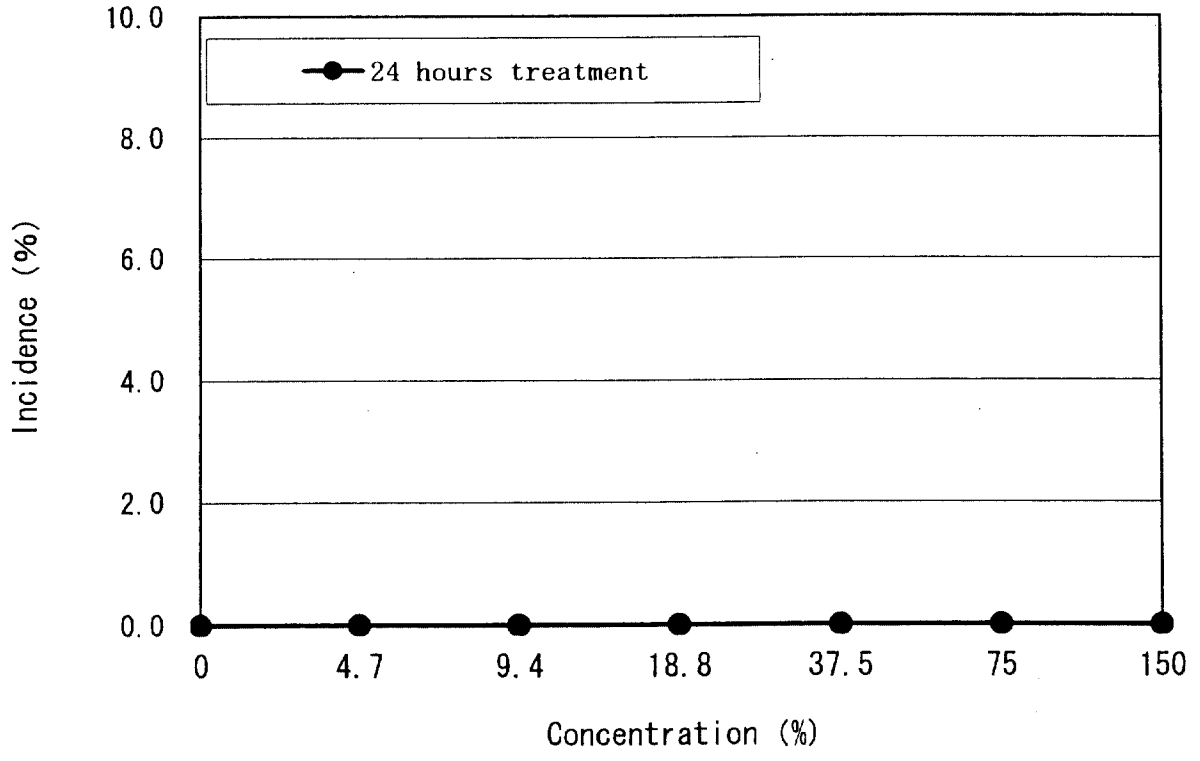

Fig. 9 Incidence of numerical aberrant cell treated with DEQUEST 2066

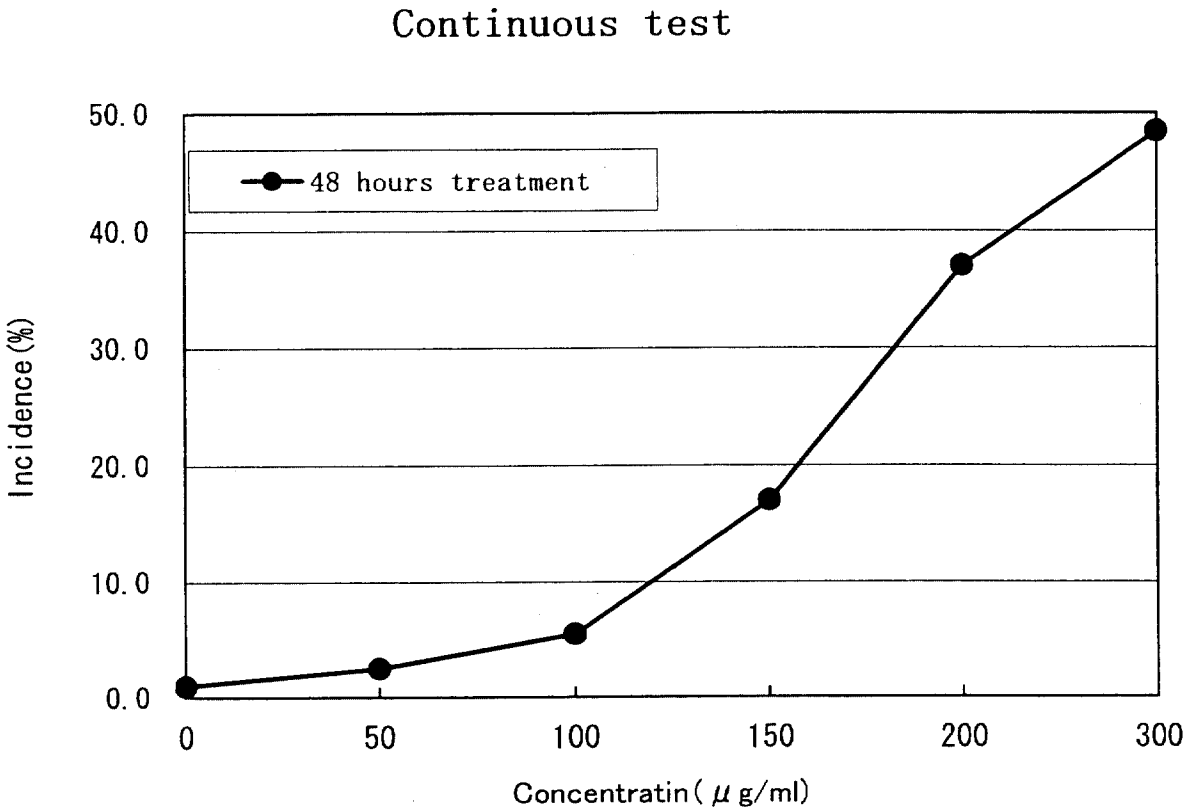

Fig. 10 Incidence of structural aberrent cell treated with DEQUEST 2066

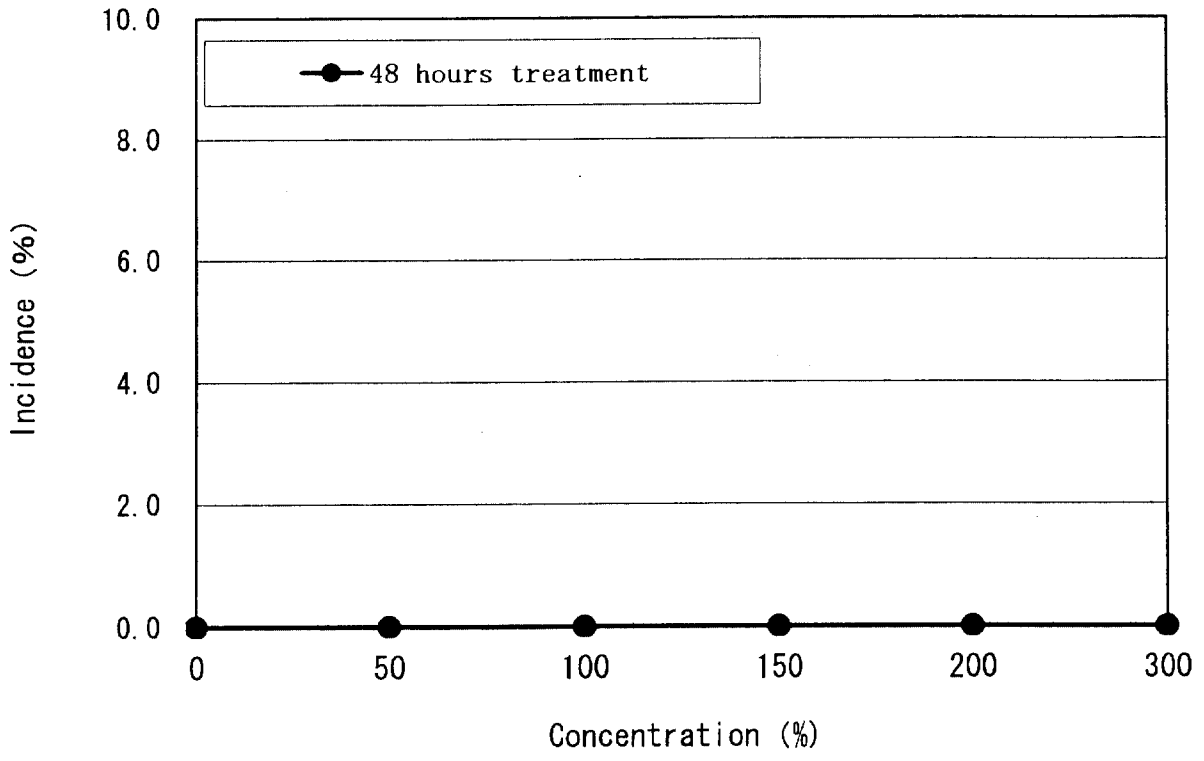

Fig. 11 Incidence of numerical aberrant cell treated with DEQUEST 2066

Photo. 1 Normal cell for the negative control

(Continuous tests, 48 hours treatment)

Photo. 2 Structural aberration cell for the treated group

(Continuous tests, 48 hours treatment 300 μ g/ml)

[The attached sheet 1]

Mitotic Index

(1)Pulse treatment tests

Without metabolic activation(6-18hr)			With metabolic activation(6-18hr)		
Treatment Concentration (μg/ml)	Number of cells	Mitotic index(%)	Treatment Concentration (μg/ml)	Number of cells	Mitotic index(%)
Negative control (Saline)	2000	7.9	Negative control (Saline)	2000	9.5
625	2000	8.3	625	2000	8.9
1250	2000	8.2	1250	2000	9.0
2500	2000	5.4	2500	2000	9.3
5000	2000	3.2	5000	2000	9.1
Positive control (MMC, 0.10)	2000	4.8	Positive control (BP, 15)	2000	8.0

(2) Continuous tests

24-0 hr			48-0 hr		
Treatment Concentration (μg/ml)	Number of cells	Mitotic index(%)	Treatment Concentration (μg/ml)	Number of cells	Mitotic index(%)
Negative control (Saline)	2000	8.4	Negative control ((Saline)	2000	9.1
4.7	2000	7.9	50	2000	7.6
9.4	2000	6.4	100	2000	3.0
18.8	2000	7.7	150	2000	2.6
37.5	2000	5.1	200	2000	2.0
75	2000	4.0	300	2000	2.1
150	2000	1.6	400	「TOX」**	
300	「TOX」*				
Positive control (MMC, 0.03)	2000	5.7	Positive control (MMC, 0.03)	2000	8.1

MMC : Mitomycin C, BP : Benzo [a] pyrene

「TOX」*: The delay of cell cycle by the test substance, metaphase cell had not obtained more than 50 cells

「TOX」**: The inhibition of cell growth by the test substance, metaphase cell had not obtained more than 50 cells